α-Methyl Substrates of Carboxypeptidase A. A Steric Probe of the Active Site†

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ABSTRACT: Although optical resolution of α-methylphenylalanine (α-Me-Phe) has been achieved by the action of carboxypeptidase A on the *N*-trifluoroacetyl derivative of the amino acid (TFA-α-Me-Phe), it is improbable that an α-methyl substrate could bind in the same orientation as glycyl-L-tyrosine, due to steric interaction of the α-methyl group with an atom in the imidazole ring of zinc ligand His-196. The kinetic parameters for TFA-α-Me-Phe and for an ester substrate bearing an α-methyl group (β-hippuryl-α-methylphenyllactic acid, HMPL) have been determined and compared to those for the appropriate nonmethylated control substrates. Both TFA-α-Me-Phe and HMPL appear to be bound nearly as well as are their respective controls, and HMPL is hydrolyzed nearly as rapidly as its control. TFA-α-Me-Phe, however, is hydrolyzed only about one-fiftieth as rapidly as is the nonmethylated substrate. These findings are consistent with the possibilities that: (1) the proposed substrate-induced conformational shift of Tyr-248 is hindered when the methylated substrates are bound; (2) the orientation in which α-methyl substrates are bound precludes optimal positioning of Tyr-248 and the scissile bond even after the rotation of Tyr-248 has occurred; (3) amides and esters are bound in different orientations, and in the amide orientation an α-methyl group is so directed as to interfere with the approach of Glu-270 to the scissile bond.

Investigation of carboxypeptidase A (CPA)[1] in this laboratory was begun to determine the utility of the enzyme in achieving optical resolution of α-methylamino acids that were to be incorporated into analogs of small peptide hormones (Turk et al., 1975). CPA was chosen because another enzyme commonly used for similar purposes, hog renal acylase I, was known to be unable to catalyze the hydrolysis of *N*-acetyl-α-methylphenylalanine (Almond et al., 1962), and the *N*-trifluoroacetyl derivatives of the amino acids were prepared since they had been shown to be far superior substrates for CPA when compared to the usually employed *N*-acetyl derivatives (Fones and Lee, 1953).

The fact that α-methylphenylalanine (α-Me-Phe) could be successfully resolved in this manner (Turk et al., 1972, 1975) prompted the question of whether one would have predicted that the enzyme would be able to bind and cleave substrates bearing an α-methyl group if the geometry of the active site were considered. The Lipscomb model of the active site, based on crystallographic studies of the Gly-Tyr complex of CPA (Lipscomb et al., 1968; Quiocho and Lipscomb, 1971), exhibits the following chief features as shown in Figure 1: (1) the Zn ligands are His-196, His-69, Glu-72, and the carbonyl oxygen of the substrate's ultimate peptide bond; (2) the guanidinium of Arg-145 forms a salt bridge with the free carboxylate of the substrate; (3) the phenolic hydroxyl of Tyr-248 approximates the nitrogen of the susceptible peptide bond; (4) the carboxylate of Glu-270 approximates the carbonyl carbon of the susceptible peptide bond; (5) the side chain of the substrate's C-terminal residue fits into a hydrophobic pocket of the enzyme.

A computer-simulated model (Marshall et al., 1972) of the enzyme–substrate complex based on the Lipscomb coordinates was generated, and it was found that if one attaches a methyl group to the Tyr α carbon of the Gly-Tyr in the model as shown in Figure 2, the distance between the centers of the carbon of the methyl group and atom ND1 of the imidazole of Zn ligand His-196 is 2.73 Å. The sum of the van der Waals radii of a CH_3 and an NH group is generally estimated to be between 3.05 and 3.50 Å (Nemethy and Scheraga, 1965). The Ramachandran minimum distance between nonbonded atoms of carbon and nitrogen, however, is estimated to be only 2.80–2.90 Å, but this estimate does not take into account the steric effect of protons attached to either of the atoms (Ramachandran and Sasisekharan, 1968).

This apparent steric interaction was thought to be of interest, especially in view of the recent controversy regarding the relationship of the crystalline and solution conformations of the enzyme (Johansen and Vallee, 1971; Quiocho et al., 1972). It was therefore decided to examine the kinetics of the cleavage of *N*-trifluoroacetyl-α-methylphenylalanine (TFA-α-Me-Phe) relative to those of *N*-trifluoroacetylphenylalanine (TFA-Phe) in order to assess the degree of interference of the methyl group on binding and catalysis.

Since CPA also possesses an esterase activity that can occasionally be affected differently from the amidase activity (Coleman and Vallee, 1960; Simpson and Vallee, 1966; Riordan et al., 1967), it was also decided to prepare and to examine the kinetics of an ester substrate bearing an α-methyl group. The ester chosen was β-hippuryl-α-methylphenyllactic acid (HMPL), principally because the nonmethylated analog, β-hippurylphenyllactic acid (HPLA), is considered a standard substrate for CPA and is commer-

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[1] Abbreviations used are: CPA, carboxypeptidase A; TFA, trifluoroacetyl; HMPL, hippuryl-α-methylphenyllactic acid; HPLA, β-hippurylphenyllactic acid.

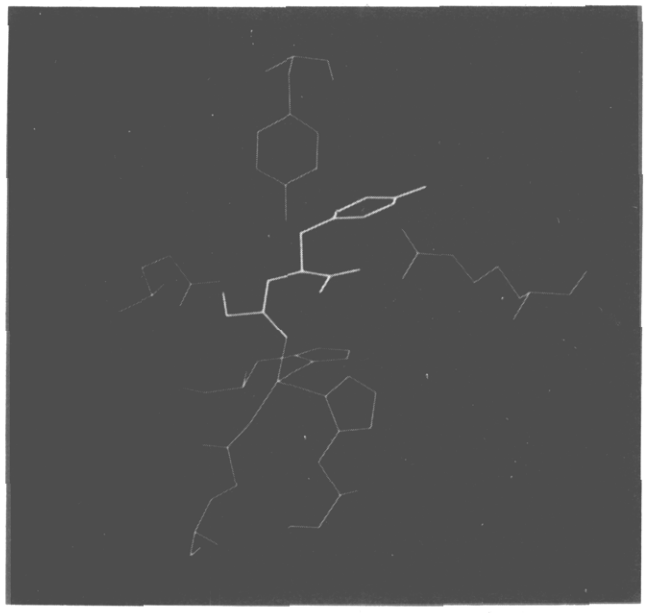

FIGURE 1: A computer-simulated model of the Gly-Tyr complex of CPA displaying some key features of the active site as determined by Lipscomb and coworkers. The inhibitor appears in the center and its bonds are intensified relative to those of the enzyme. Below the substrate are located the zinc ion and its ligands. To the right is Agr-145; to the left is Glu-278; and above the substrate is Tyr-248.

cially available. The results of the kinetic studies on these two pairs of synthetic substrates are the substance of this report.

Materials and Methods

Substrates. Melting points were determined in open capillaries and are uncorrected. Thin-layer chromotography (TLC) was performed on each new compound on silica gel G plates (Brinkman) in three solvent systems: chloroform–methanol–acetic acid (85:10:5), 1-butanol–acetic acid–water (1:1:1), and 1-butanol–pyridine–acetic acid–water (15:10:3:12). The R_f values in these systems will be designated R_{f1}, R_{f2}, and R_{f3} respectively. Elemental analyses were performed by PCR, Inc., Gainesville, Fla.

N-Trifluoroacetylphenylalanine (Fones, 1952; Schallenberg and Calvin, 1955) and *N*-trifluoroacetyl-α-methylphenylalanine (Turk et al., 1972, 1975) were prepared from phenylalanine and α-methylphenylalanine (Almond et al., 1962; Stein et al., 1955) by the trifluoroacetylation procedure of Weygand and Geiger (1955).

α-Methylphenyllactic acid was prepared from phenylacetone (Aldrich Chemical Co.) in 25% yield via the cyanohydrin route of Elliel and Freeman (1964). Physical characterization of the compound revealed mp 92–93°, R_{f1} = 0.34, R_{f2} = 0.78, R_{f3} = 0.66. The IR spectrum will be summarized by citing the major absorption maxima in wave numbers, followed by the approximate intensity of the absorption relative to that for the C=O stretch (at 1720 cm^{-1}): 3400, 0.76; 2900, 0.52; 2585, 0.29; 1720, 1.00; 1470, 0.20; 1260, 0.50; 1180, 1.03; 1137, 0.57; 1090, 0.60; 950, 0.14; 900, 0.14; 792, 0.50; 758, 0.10; 740, 0.13; 690, 0.50. Among the major fragments observed in the mass spectrum were the molecular ion $[C_6H_5CH_2C(OH)(CH_3)CO_2H]^+$, M = 180; $[C_6H_5CH = (CH_3)CO_2H]^+$, M = 162; $[C_6H_5CH_2C(OH)CH_3]^+$, M = 125; $[C_6H_5CH = CCH_3]^+$, M = 117; $[C_6H_5CH_3]^+$, M = 92; $[C_6H_5CH_2]^+$, M = 91; $[C_6H_5]^+$, M = 77; $[CO_2]^+$, M = 44. *Anal.* Calcd for $C_{16}H_{12}O_3$: C, 66.70; H, 6.67; N, 0.00. Found: C, 66.72; H, 6.89; N, 0.00.

β-Hippurylphenyllactic acid was purchased from Sigma Chemical Co. β-Hippuryl-α-methylphenyllactic acid was prepared in 92% yield from 2-phenyl-5-oxazalone (Cornforth, 1949; Carter et al., 1953) and α-methylphenyllactic acid by the esterification procedure of Kaiser and Carson (1965) and of Whitaker et al. (1966). Physical characterization revealed mp 143–144°, R_{f1} = 0.28, R_{f2} = 0.81, R_{f3} = 0.68. The IR spectrum was essentially identical with that of an equimolar mixture of hippuric acid and α-methylphenyllactic acid except that, as expected, a strong new maximum was observed in the range 1200–1230 cm^{-1} due

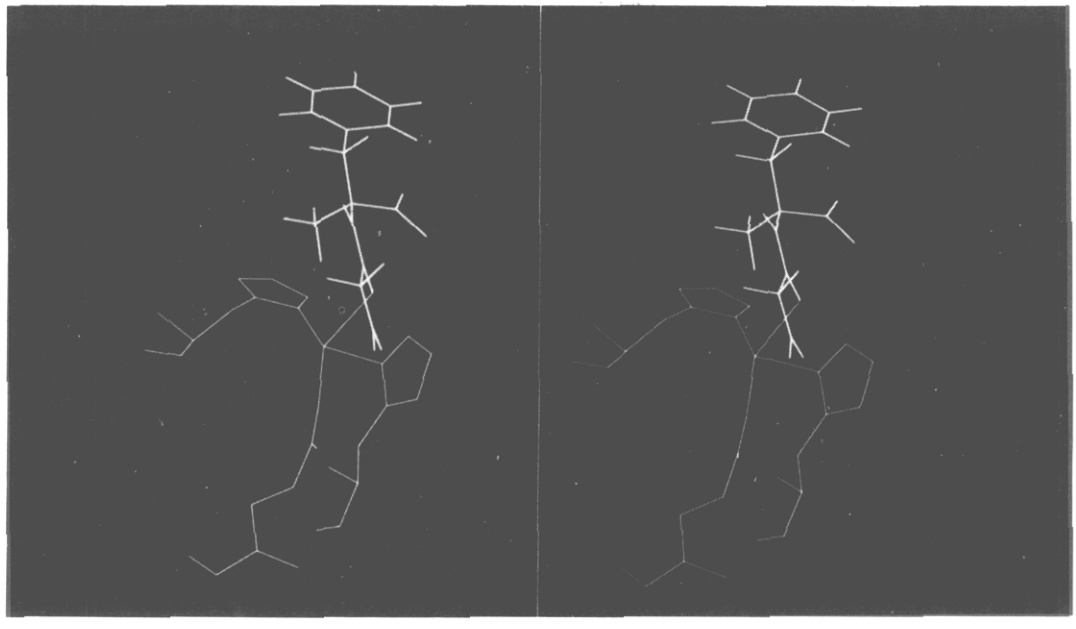

FIGURE 2: Stereo views of the relationship of Gly-L-α-Me-Phe to the zinc ligands of CPA based on the Lipscomb model. Again the substrate is intensified and it appears at the top. Substrate protons are included. Below the substrate are located His-69 at the right, Glu-72 in the lower center, and His-196 to the left. Note that the methyl group of the substrate points directly toward atom ND1 in the imidazole ring of His-196.

Table I[a]

Substrate	K_M (M)	k_{cat} ($min^{-1} \times 10^{-2}$)
HPLA	8.9×10^{-5}	370
HMPL	1.1×10^{-4}	210
TFA-Phe	2.2×10^{-3}	132
TFA-α-Me-Phe	2.3×10^{-3}	3.17

[a] The values tabulated are for the γ form of the enzyme. The values for both α and β forms were also determined and were different from the values displayed here by less than 10%.

to the stretch of the newly created C–O bond (Cross, 1960). The spectrum will be summarized as above: 3390, 0.52; 3130, 0.37; 1750, 1.00; 1635, 0.98; 1560, 0.83; 1500, 0.35; 1470, 0.28; 1415, 0.42; 1380, 0.63; 1325, 0.35; 1220, 1.07; 1115, 0.56; 1105, 0.56; 1035, 0.16; 1010, 0.14; 760, 0.42; 730, 0.33; 700, 0.72. No molecular ion could be identified in the mass spectrum, but fragments were observed which probably derived from the hippurate moiety ($[C_6H_5CONHCH_2CO]^+$, M = 162; $[C_6H_5CONHCH_2]^+$, M = 134; $[C_6H_5CO]^+$, M = 105) together with a fragment likely to have derived from the α-methylphenyllactate moiety ($[C_6H_5CH_2]^+$, M = 91). Thin-layer chromatography demonstrated that the product was contaminated neither with unreacted hippuric acid (mp 189–190°, R_{f1} = 0.11, R_{f2} = 0.72, R_{f3} = 0.60) nor with unreacted α-methylphenyllactic acid, and the IR spectrum demonstrated the absence of contaminating oxazalone since it lacked the sharp absorbance at 1832 cm^{-1} characteristic of the azolactones (Bodansky and Ondetti, 1966). *Anal.* Calcd for $C_{19}H_{19}O_5N$: C, 67.01; H, 5.67; N, 4.10. Found: C, 66.51; H, 5.87; N, 4.00.

Kinetics. Purified α, β, and γ forms of CPA were generously supplied from the laboratory of Dr. Hans Neurath by Dr. P. H. Petra. All reactions were run at 25° in 0.05 *M* Tris-HCl at pH 7.5 with 0.5 *M* NaCl and 0.02 *M* LiCl. The CPA concentrations of the stock solutions were determined by the optical density at 278 nm (Hirs, 1967), and the concentration in the reaction vessel was adjusted to 10^{-10} *M* for esterase runs and to 10^{-9}–10^{-7} *M* for amidase runs. Substrate concentrations were varied between 3×10^{-4} and 3×10^{-5} *M* in the case of esters and between 5×10^{-3} and 1×10^{-4} *M* in the case of amides. All substrates except TFA-Phe were racemic, and the above concentrations refer to the L isomer. Esterase activity was followed spectrophotometrically as the accumulation of hippurate at 245 nm (Davies et al., 1968), and amidase activity was followed by observing the change in optical rotation at 265 nm. Kinetic parameters were obtained from double-reciprocal plots (Lineweaver and Burk, 1934), which were linear in the concentration ranges indicated.

Results and Discussion

Table I summarizes the results of the kinetic studies. The fact that the k_{cat} values for TFA-Phe and HPLA are of the same order of magnitude may seem surprising in view of the facts that esters are normally cleaved five to ten times more rapidly than peptides (Snoke and Neurath, 1949) and peptides 1000 times more rapidly than *N*-acetylamino acids (Bergman and Fruton, 1937). Under conditions of saturation, however, *N*-trifluoroacetylamino acids are cleaved 3000–5000 times more rapidly than are *N*-acetylamino acids (Fones and Lee, 1953). These findings predict that the k_{cat} for TFA-Phe should be lower than that for HPLA by a factor of 2–4, which is the observed result. Since *N*-acetylamino acids do not exhibit markedly higher K_M values than peptides (Bergman and Fruton, 1937), the fact that the K_M for TFA-Phe falls within the range normally observed for peptides is also reasonable. Finally, of the four substrates which we examined, only the kinetic parameters for HPLA have been previously reported to our knowledge, and our values agree well with the earlier results (Davies et al., 1968).

There are two fundamental observations of interest here. The first is that the k_{cat} of HMPL is depressed relative to that of HPLA to a far smaller extent than is that of TFA-α-Me-Phe relative to that of TFA-Phe. This observation complements a number of experiments involving chemical modification of the enzyme which result in reduced peptidase but not esterase activity (Coleman and Vallee, 1960; Simpson and Vallee, 1966; Riordan et al., 1967). To our knowledge no modification of enzyme or substrate has resulted in a selective reduction of esterase activity.

Second, the K_M for TFA-α-Me-Phe is much less affected relative to that for TFA-Phe than is the k_{cat}. A trivial explanation of these observations is that TFA-D-α-Me-Phe is a noncompetitive inhibitor of the hydrolysis of the L isomer, but this is unlikely since the presence of an equimolar concentration of TFA-D-α-Me-Phe does not affect the rate of catalytic hydrolysis of TFA-L-Phe. Rather, it appears that an α-methyl group on an amide substrate produces an intrinsic depression of the k_{cat} while inducing no change in the K_M. Since the K_M for a substrate of CPA is generally considered to be its binding constant (Auld and Vallee, 1970; Auld et al., 1972), this implies that the α-methyl group does not interfere with the binding of the amide substrate even though it does cause a substantial decrease in the rate of catalysis. One interpretation of these observations is that the methyl group blocks the approach to the susceptible peptide bond by a group much more strongly involved in catalysis than in binding.

A summary of the candidate groups in the active site to which this interpretation might be applied has been provided in the introduction to this paper. Only three of the interactions discussed there need be invoked to provide sufficient constraints on binding to assure stereoselectivity: (1) the salt bridge between the substrate carboxylate and the guanidinium of Arg-145; (2) the hydrophobic pocket in which the side chain of the C-terminal residue sits; and (3) the ligand relationship of the zinc atom and the carbonyl oxygen of the substrate as shown in Figure 3.

It is clear that only three such constraints need be imposed since one may orient two enantiomers so as to superimpose any two of the four distinct groups around an asymmetric carbon, though there exists no orientation in which more than two of the groups can be superimposed. Thus, one could orient TFA-D-Phe so that the side chain would fit into the hydrophobic pocket and the substrate carboxylate would point toward Arg-145, but then the carbonyl oxygen would not point in the direction of the zinc atom. In order to point the carbonyl oxygen in that direction, one would need to reorient the molecule so as to remove the side chain from the pocket or to remove the carboxylate from its interaction with Arg-145. By binding only molecules which satisfy all three of these conditions at once, the enzyme is able to distinguish between L and D isomers.

This leaves two groups in the active site which need not participate in binding but which are thought to participate strongly in catalysis, namely Glu-270 and Tyr-248. The

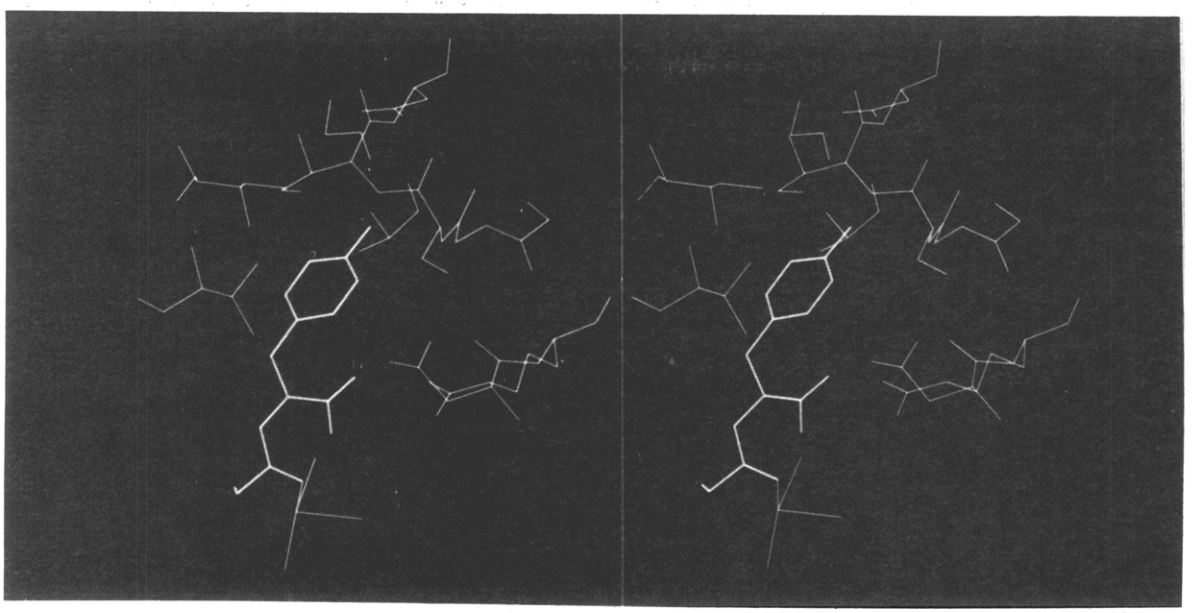

FIGURE 3: Stereo views depicting the contacts required to assure stereoselectivity of CPA action. The substrate is again intensified and appears in the center of the picture. Above is located the hydrophobic pocket which surrounds the aromatic ring of the substrate; to the right is Arg-145, whose guanidinium group engages in a salt linkage with the substrate carboxylate. Below, the ligand relationship of the zinc ion and the carbonyl oxygen of the substrate is depicted.

carboxylate of Glu-270 is thought to initiate a nucleophilic attack on the carbonyl carbon of the susceptible peptide bond (Lipscomb et al., 1968; Quiocho and Lipscomb, 1971). Specific chemical modification of this group results in a parallel decrease in both amidase and esterase activities (Petra and Neurath, 1971; Haas and Neurath, 1971a,b), however, and so a hypothetical blockade of access by this group to the susceptible peptide bond would not provide a ready explanation of our observations.

A more plausible candidate is Tyr-248, whose phenolic hydroxyl is thought to participate in catalysis by donating a proton to the nitrogen of the susceptible peptide bond (Lipscomb et al., 1968; Quiocho and Lipscomb, 1971). Specific modification of this group does result in a selective abolition of peptidase activity (Simpson and Vallee, 1966; Riordan et al., 1967). It is unlikely, however, that the α-methyl group interferes directly with the approximation of Tyr-248 to the nitrogen of the peptide bond, since it points in the wrong direction to do so. A more subtle possibility is that when the α-methyl substrate is bound so as to avoid steric interaction with His-196, it is oriented either so as to hinder the proposed rotation of Tyr-248 that occurs on binding (Lipscomb et al., 1968; Quiocho and Lipscomb, 1971) or so as to preclude optimal positioning of the phenolic hydroxyl of Tyr-248 and the nitrogen of the peptide bond even after the rotation of Tyr-248 has occurred.

In order to accept that hypothesis, one must account in some way for the fact that there is some residual catalytic hydrolysis of TFA-α-Me-Phe. One possibility is that the enzyme retains some catalytic effectiveness even without the participation of Tyr-248 by virtue of the fact that it can still bind the substrate and initiate a nucleophilic attack with Glu-270. A water molecule might then perform the normal function of Tyr-248, though not as rapidly. This idea is lent some plausibility by the fact that even CPA preparations in which Tyr-248 has been chemically modified do retain small amounts of catalytic activity (Simpson and Vallee, 1966; Riordan et al., 1967), although it could be argued that this is due to contaminating native enzyme that has escaped modification. It is also possible that Tyr-248 is strictly required to participate in catalysis and that rotation of that residue can still occur with the α-methyl substrates. In the binding orientation of an α-methyl amide substrate, however, the energetic barrier to that rotation might be raised sufficiently so that it would occur much more slowly and thus become the rate-limiting step in catalysis.

One is still left with the fact that despite the proposed shift in binding orientation for the α-methyl substrates, they exhibit K_M values nearly identical with their nonmethylated controls. This suggests that the major binding interactions are identical for both types of substrate and that there may be no major change in binding orientation of the α-methyl substrates. To accept the idea that methylated and nonmethylated substrates are bound in the same orientation, however, one would have to concede that this orientation is different from that observed for Gly-Tyr, since in that orientation the α-methyl substrates would encounter the steric interference previously described. This would imply that the Gly-Tyr complex of CPA does not reflect the productive substrate binding orientation, a notion that is given some support by the low rates of catalytic hydrolysis observed for Gly-Tyr (Yanari and Mitz, 1957a,b). The resistance of this substrate to degradation by CPA has been attributed to an unfavorable interaction of its α-amino group with Glu-270, however (Lipscomb et al., 1968; Quiocho and Lipscomb, 1971), and there is no clear evidence that the binding orientation of Gly-Tyr is nonproductive in other respects, particularly as regards the disposition of the Tyr α carbon and its substituents.

In any case, the explanations so far presented for the low k_{cat} observed for TFA-α-Me-Phe rest on the supposition that this substrate is bound in an anomalous orientation. This assumes that the substrate is a poor model for a discussion of productive binding. It is possible to rationalize the low rates of catalysis observed for TFA-α-Me-Phe without assuming that it is bound in an anomalous orientation, however, if one supposes that its α-methyl group interferes directly with the approach of a catalytic function to the susceptible peptide bond. The difficulty with this argument, as has been noted, is that in order for the α-methyl group to

cause direct steric interference with Tyr-248, the substrate would have to be oriented very differently from the inhibitor in the Gly-Tyr complex of CPA. It should be pointed out that a somewhat less drastic reorientation would be required to obtain direct steric interference with Glu-270, but it will be recalled that the carboxylate function of that residue is thought to participate both in amidase and esterase activities while the α-methyl group interferes with only the amidase activity. It could well be that esters and amides are bound in different orientations, however, and that only in the amide binding orientation is the methyl group so directed as to interfere with the approach of Glu-270 to the scissile bond. It is not unreasonable to consider the possibility of different binding orientations for esters and amides, since the fact that the K_M values for esters are generally two orders of magnitude lower than those for amides (Snoke and Neurath, 1949) suggests that rather different binding contacts could be involved, as does the observation that replacing the zinc ion in the enzyme with a cadmium ion results in reduced affinity for esters without significant alteration of the affinity for peptides (Auld and Holmquist, 1974). In fact, these authors suggest that in the case of ester substrates the free carboxylate rather than the carbonyl oxygen of the scissile bond may coordinate with the zinc ion of CPA.

On balance it seems fair to say that our data can be reconciled with existing crystallographic information about the active site of CPA by assuming that the Gly-Tyr complex of CPA does reflect accurately the principal features of amide binding and that substrates bearing an α-methyl group are bound in a somewhat different orientation, in which the major binding contacts are retained, although steric interaction with His-196 is avoided by displacing the substrate so as to produce a new binding orientation in which it is more difficult for Tyr-248 to achieve the required proximity to the scissile bond. As the foregoing discussion has indicated, however, alternate explanations are possible. One of these explanations assumes a different binding orientation for ester and amide substrates and involves direct steric interference of the α-methyl group of amide substrates with the approach of the carboxylate of Glu-270 to the carbonyl carbon of the susceptible peptide bond.

Acknowledgments

We are grateful to Dr. H. Neurath for the gift of the enzyme used in these studies, to Dr. R. Bradshaw for suggesting that we prepare an ester substrate and for a critical reading of the manuscript, to Dr. W. Sherman for aid in interpretation of the mass spectra of the newly synthesized compound, and to Dr. C. David Barry and Dr. Ed Czerwinski for assistance in the preparation of the figures.

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